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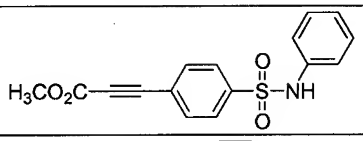
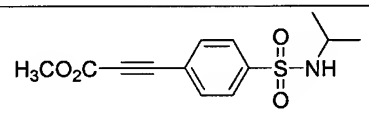
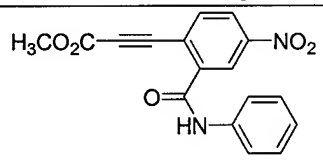
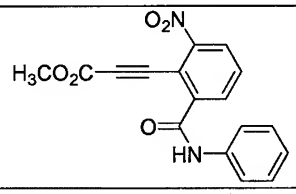
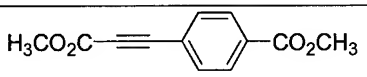
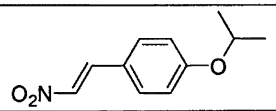
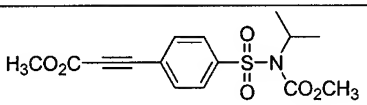
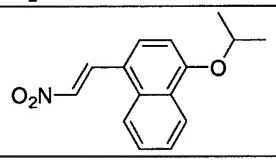
Task 1. Optimization of the activity of the lead compounds

Our studies in the first two years have led to the identification of a number of promising "lead" compounds. Among these initial lead compounds, **B-17** has excellent activity in inhibition of erbB-2 auto-phospharylation and inhibition of cell proliferation using MDA-453 human breast cancer cells with erbB-2 overexpression. Furthermore, this compound appears to also be quite selective of breast cancer cells and model cells with EGFR overexpression but not erbB-2 overexpression (see below). Accordingly, **B-17** represents a quite promising lead compound for further modifications.

Based upon our predicted three-dimensional binding model for **B-17** in complex with erbB-2, we have designed a number of new analogues of **B-17**. To date, we have completed the synthesis of 10 new analogues, whose structures are provided in **Table 1**. We are currently evaluating their activity in inhibition of erbB-2 auto-phospharylation and inhibition of cell proliferation using MDA-453 human breast cancer cells with erbB-2 overexpression. We expect that more potent and selective erbB-2 inhibitors may be obtained from these newly designed and synthesized analogues of **B-17**.

Table 1. Newly designed and synthesized analogues of B-17.

Compound	Structure	Compound	Structure
Y-6-14		**Y-6-46-2**	
Y-6-17		**Y-6-23-1**	
Y-6-11		**Y-6-55**	
Y-6-39-2		**Y-6-99**	

Task 2. Biological confirmation of potential kinase inhibitors and molecular mechanism studies

We have shown in our last yearly report (2002) that a lead compound (**B-17**) identified from our computational structure-based database searching appeared to demonstrate promising activity in inhibition of auto-phosphorylation in MDA-MB-453 breast cancer cells with overexpression of erbB-2 and selectivity in MDA-MB-468 breast cancer cells with overexpression of EGFR but not erbB-2.

Furthermore, we demonstrated that **B-17** potently inhibited cell growth in MDA-MB-453 breast cancer cells and has much less activity in MDA-MB-468 breast cancer cells. Our initial data suggested that B-17 may be a promising erbB-2 selective kinase inhibitor. Toward further characterization of its activity, selectivity and molecular mechanism of action, we have carried out additional studies on this promising lead compound. Some of the results are summarized in this report.

Task 2.a. *Further investigation of the activity and selectivity of B-17 in human breast cancer and model cell lines.*

We have used two additional human breast cancer cell lines, T47D and 47T1LZ1, which overexpress erbB-2/Her-2, to evaluate the activity of **B-17**. **B-17** potently inhibits the cell growth in these two cell lines with IC_{50} values of 0.7 and 0.7 μM, respectively (**Table 2**). To further evaluate the selectivity of **B-17**, we have tested **B-17** in A431 cells, which have a high level of EGFR but a low level of erbB-2 expression and found that **B-17** has an IC_{50} value of greater than 2.5 μM. Similarly, in MDA-MB-231 cells, which have a low level of EGFR and erbB-2, B-17 has an IC_{50} value greater than 2.5 μM. Thus, **B-17** potently inhibits cell growth in human breast cancer cells with a high level of erbB-2 and displays selectivity in breast cancer cells with a low level of erbB-2 but a high level of EGFR, or neither protein overexpression.

Table 2. Inhibitor effects of B-17 in cultures of erbB-2/HER-2/neu -overexpressing *versus* EGF receptor human cancer cell lines

		IC_{50}			IC_{50}
Cell line	Overexpression	(μM)	Cell line	Overexpression	(μM)
			A431	EGFR	>2.5
MDA453	HER-2/erbB-2	0.29	MDA-MB468	EGFR	>2.5
T47D	HER-2/ erbB-2	0.7	NIH3T3/EGFR	EGFR	>2.5
47T1LZ1	HER-2/ erbB-2	0.7			
NIH3T3/erbB2	HER-2/ erbB-2	1.2	MDA-MB-231 (2LMP)	Neither	>2.5

Human cancer cell lines have many genetic differences between them in addition to their differences in levels of erbB-2 and EGFR proteins. To overcome this limitation, we also evaluated the activity and selectivity of B-17 in NIH3T3 cells transfected with either erbB-2 or EGFR. B-17 potently inhibits cell growth in NIH3T3 cells transfected with erbB-2 with an IC_{50} value of 1.2 μM and is less active in NIH3T3 cells transfected with EGFR. Taken together, our results demonstrated that B-17 potently inhibits cell growth in human breast cancer and model cell lines with high levels of erbB-2 and has a relative selectivity in breast cancer cells and model cells with low levels of erbB-2.

Task 2.b. *Investigation of the molecular mechanism of action of* ***B-17*** *in vivo*

We have demonstrated that B-17 has potent anti-tumor activity in inhibition of tumor growth in the BT-474/M1 xenograft model of human breast cancer with overexpression of erbB-2. To gain further insight on its molecular mechanism of action *in vivo*, we have investigated the effect of B-17 on the phosphorylation of erbB-2, its down-stream MAP kinase and total erbB-2 protein in BT-474/M1 cells. The results are shown in **Figure 1**. Briefly, mice bearing BT-474/M1 tumors were treated with B-17 (30 mg/kg) or with vehicle control, and the phosphorylated erbB-2 and MAPK as well as total erbB-2 protein were probed using specific antibodies for each protein. We found that while there was no apparent change in the total amount of erbB-2 protein, the phosphorylated erbB-2 protein was significantly decreased as compared to the vehicle control tumors. Furthermore, the phosphorylated MAPK protein, a downstream target of erbB-2, was also significantly decreased as compared to the vehicle control tumors. These results were consistent with our in vitro results, which showed that B-17 directly binds to erbB-2 protein and inhibits its kinase activity. Our *in vivo* results further suggested that the in vivo anti-tumor activity of B-17 is unlikely due to down-regulation of the erbB-2 protein level in cancer cells, but instead through direct inhibition of the kinase activity of erbB-2 protein.

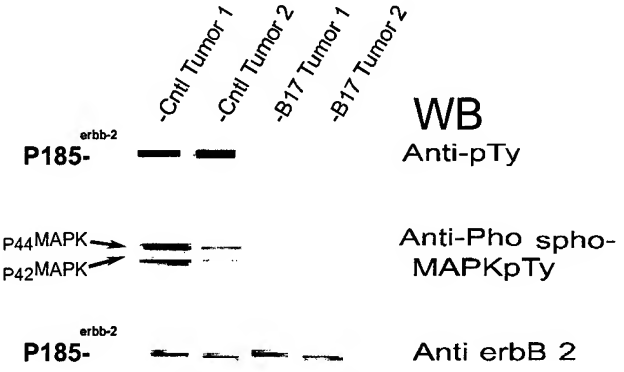


Figure 1. *in vivo* Effects of **B-17**on Phosphorylation of erbB-2, MAP kinase and total erbB-2 in BT-474/M1 cells (24 Hours after Intraperitonia Treatment).

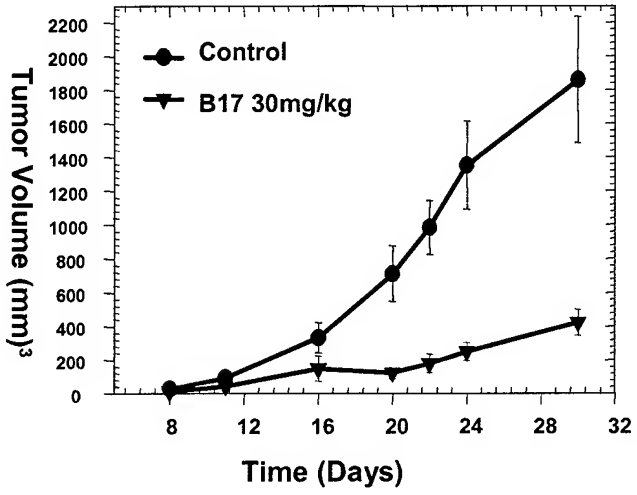
Task 3. Further evaluation of in vivo antitumor activity of B-17

We have further evaluated the anti-tumor activity of B-17 *in vivo* using an additional human breast cancer cell line (MDA-316/DYT2) with erbB-2 overexpression in the xenograft model (**Figure 2**). In the MDA-316/DYT2 (human breast carcinoma cells, ER positive and subclone DYT2), we

administered the B-17 on the second day after cell inoculation. With B-17 injected at 30mg/kg twice a week for three weeks, 80% tumor growth inhibition (8-10 tumors in each group) was achieved in the treated group of mice as compared to the control group with no treatment. It is noted that none of the mice showed weight loss or any other signs of toxicity at the dose regimen used.

Taken together, our *in vivo* data with two xenograft models of human breast cancer (BT-474/M1 and MDA-361/DYT2) demonstrated that B-17 has potent anti-tumor activity *in vivo,* and inhibition of the erbB-2 kinase activity using a fairly potent and erbB-2 specific small molecule kinase inhibitor has the therapeutic potential for the treatment of cancers with erbB-2 overexpression.

Figure 2. *In vivo* inhibition of tumor growth by **B-17** in MDA-361/DYT2 human breast cancer xenograft model in mice.



Project Plan for Year 3.

- We will complete the characterization of these newly designed and synthesized analogues of B-17 as shown in Table 1 to identify more potent and/or more selective erbB-2 inhibitors (**Tasks 1** and **2**).
- Using two animal models of human breast cancer cell lines, we have shown that **B-17** has a potent antitumor activity *in vivo* in inhibition of tumor growth. Our results suggest that B-17 represents a promising lead compound *in vivo*. We plan to evaluate an additional 1-2 of the most promising new erbB-2 inhibitors in animal models of human breast cancer and identify the most promising erbB-2 inhibitor(s) for advanced preclinical studies.
- We plan to complete and submit 2-3 manuscripts on this project. One manuscript on the discovery of **B-17** is nearly completed.